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BASIC SCIENCE

High-Density Lipoprotein Increases the Abundance of eNOS Protein in Human Vascular Endothelial Cells by Increasing its Half-Life

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OBJECTIVES Given the importance of endothelial nitric oxide synthase (eNOS) in regulating endothelium-dependent vasorelaxation, we investigated the effects of high-density lipoprotein in (HDL) on eNOS protein abundance in cultured human vascular endothelial cells.

BACKGROUND Endothelial dysfunction, characterized by decreased nitric oxide production, is one of the early features in the development of atherosclerosis. We have recently shown in vivo that niacin therapy increases plasma HDL concentration and improves endothelium-dependent vasorelaxation in patients with coronary artery disease.

METHODS Human vascular endothelial cells were cultured in the presence or absence of HDL or apolipoprotein (apo)A-I. The eNOS protein abundance was assessed by immunoblotting, and protein half-life was assessed by pulse-chase techniques. The eNOS messenger ribonucleic acid (mRNA) abundance was measured using real-time quantitative polymerase chain reaction.

RESULTS High density lipoprotein, or apoA-I alone, increased eNOS protein abundance by 3.5 ± 0.7 and 2.7 ± 0.5-fold, respectively ($p < 0.05$ for both). However, neither HDL nor apoA-I increased eNOS mRNA abundance. It was shown that HDL increased eNOS protein half-life up to 3.3 ± 0.2-fold ($p = 0.001$). Both HDL and apoA-I activated mitogen-actived protein-kinase and phosphatidylinositol 3-kinase (PI3K) Akt-pathways in human arterial endothelial cells, and inhibition of either of these pathways by specific pharmacologic inhibitors abolished the effect of HDL on eNOS.

CONCLUSIONS We demonstrate that HDL activates both extracellular signal-regulated kinase 1/2 (ERK1/2) and Akt, resulting in enhanced eNOS protein stability and subsequent accumulation of eNOS protein. This posttranslational regulation represents a previously unrecognized mechanism for regulating eNOS. (J Am Coll Cardiol 2003;41:2288–97) © 2003 by the American College of Cardiology Foundation

The association of low plasma high-density lipoprotein (HDL) cholesterol and coronary artery disease (CAD) has been well documented (1). We have recently shown that patients with low HDL cholesterol have abnormal endothelial function, despite having well-controlled low density lipoprotein levels (2). Endothelial cell dysfunction, characterized by decreased nitric oxide (NO) production, is one of the early pathophysiologic events in the development of atherosclerosis (3,4). Our recent study also demonstrated that raising plasma HDL cholesterol in patients with CAD improves endothelium-dependent vasorelaxation (2). We showed further that in cultured endothelial cells, HDL can increase the abundance of endothelial nitric oxide synthase (eNOS) protein, the enzyme responsible for endothelium-dependent vasorelaxation (2). The overall production of NO by eNOS is regulated by two general mechanisms. The activity of the enzyme is regulated by kinase-mediated phosphorylation (5–7), by alteration of its intracellular localization (8,9), and by alterations in eNOS protein abundance. The abundance of eNOS protein is controlled by alterations in gene transcription and/or messenger ribonucleic acid (mRNA) stability (10–12). The current study focused on understanding the mechanisms by which HDL increases eNOS protein abundance.

MATERIALS AND METHODS

Lipoprotein isolation and apolipoprotein A-I. High density lipoproteins were isolated from the plasma of healthy volunteers by sequential ultracentifugation as previously described (2). Protease inhibitors and EDTA were added to the plasma, which was maintained under aseptic conditions at +4°C. The HDL was isolated at the density of 1.09 to 1.21 g/ml after first removing lower-density lipoproteins by separate ultracentrifugation, followed by an additional wash run at 1.21 g/ml. Finally, HDL was

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Abbreviations and Acronyms

apo = apolipoprotein
CAD = coronary artery disease
eNOS = endothelial nitric oxide synthase
ERK1/2 = extracellular signal-regulated kinase 1/2
HDL = high-density lipoprotein
PI3K = phosphatidylinositol 3-kinase
RT–PCR = real-time quantitative polymerase chain reaction

dialyzed with several changes against phosphate-buffered saline (PBS). The composition of the isolated HDL was analyzed colorometrically using a Kone-specific selective chemistry analyzer (Kone, Oy, Finland) and kits from Boehringer and Wako Chemicals according to the manufacturer's instructions. This contained, on average, 8.7 mg/ml cholesterol and 28.4 mg/ml protein. It also contained 1.5 mmol/l triglyceride and 18.4 mmol/l phospholipids. Purified human apoA-I was purchased from ICN Pharmaceuticals, Costa Mesa, California. The stated concentrations of HDL used in experiments refer to the protein content.

Cell culture. The EA.hy926 cells, immortalized human aortic endothelial cells, the kind gift of Dr. Cora-Jean Edgell (University of North Carolina), were cultured in Dulbecco's modified eagle medium (DMEM). Human aortic endothelial cells (HAEC), purchased from Clontech (Palo Alto, California) were grown in M199, each as described (2). For each of the protocols described below, the cells were grown to 80% confluence and maintained in serum-and phenol red-free media for 36 h before treatment as indicated for each experiment. Cells were then harvested with triton-based lysis buffer.

Western blot analysis and antibodies. Endothelial cell lysates (20 μg of protein/sample) were separated by SDS–PAGE and transferred onto nitrocellulose membranes, which were probed with specific antibodies. Anti-eNOS antibody was purchased from Transduction Laboratories (Lexington, Kentucky) and anti-beta-actin was from Sigma-Aldrich (St. Louis, Missouri). Antibodies against total extracellular signal-regulated kinase 1/2 (ERK1/2), phospho-ERK1/2 (pERK1/2), threonine307 phospho-Akt (pAkt), and total Akt were from Cell Signaling (Beverly, Massachusetts). The horseradish peroxidase-labeled secondary antibodies were purchased from Amersham-Pharmacia (Piscataway, New Jersey). Detection of protein bands was performed by enhanced chemiluminescence (Amersham-Pharmacia) and the resulting films were subjected to spot densitometry. For all experiments, quantification of the abundance of the protein of interest was normalized to the abundance of beta-actin, total ERK1/2, or Akt.

Real-time quantitative polymerase chain reaction. Both HAEC and EA.hy926 cells were incubated with HDL (50 μg/ml), apoA-I (50 μg/ml), 17β-estradiol (10 nmol/l) or vehicle control for 24 h. Total cellular RNA was isolated using the RNeasy Mini kit (Qiagen, Valencia, California) according to the manufacturer's recommendations. The cDNA was produced from 1 μg of total cellular RNA by reverse transcription using Taqman Reverse Transcription reagents (Applied Biosystems) as instructed by the manufacturer. Negative controls were provided by performing an identical reaction in the absence of reverse transcriptase or template. Real-time quantitative polymerase chain reaction (RT–PCR) was performed in the Perkin Elmer Biosystems Gene Amp 7700 sequence detection system with SYBR green PCR Master Mix (Applied Biosystems) according to the manufacturer's recommendations. Ten nanograms of cDNA from RT reactions were used as a template, and the reactions were carried out in the volume of 50 μl in triplicates. The sense and antisense primers for beta-actin primers were 5′AGCGGGAAATCGTGCGTGAC and 5′TCCATGCCCAGGAAGGAAGG, and for eNOS 5′CTTTGCTCGTGCCGTGGACA and 5′GCCCTCGTGGACTTGCTGCT, respectively. Reactions were first incubated at 50°C for 2 min to activate the urasil N′-glycosylase and then for 10 min at 95°C to inactivate the urasil N′-glycosylase and activate the Amplitaq Gold polymerase. Forty cycles of PCR consisting of denaturation at 95°C for 15 s and annealing and extension for 1 min at 60°C were performed. To ensure the quantitative nature of the results, serial dilutions of control cDNA were analyzed to confirm that experiments were performed within the linear range of the assay.

Data from RT–PCR were analyzed by standard methodology as previously described (13). The data were plotted as fluorescence signal from each sample versus the cycle number. The threshold value was set at the midpoint of this logarithmic fluorescence signal against cycle number plot. The threshold cycle (C_T) indicates the fractional cycle number at which the amount of each amplified eNOS/beta-actin mRNA reaches the fixed threshold. The fold-change in eNOS mRNA abundance was determined by the equation:

$$\text{Fold change} = 2^{-\Delta\Delta C_T}, \text{ where } \Delta\Delta C_T = (C_{T,eNOS} - C_{T,\beta\text{-actin}})$$

$$\text{control} - (C_{T,eNOS} - C_{T,\beta\text{-actin}}) \text{ target}.$$

Quantification of eNOS mRNA half-life. The EA.hy926 cells were cultured as described above and incubated with 1 μmol/l actinomycin D for 1 h. Either HDL (50 μg/ml) or PBS was then added to the cultures. Cells were harvested after 0, 2.5, or 11 h, and total cellular RNA was isolated using the RNeasy Mini kit (Qiagen). The RT–PCR analysis of eNOS mRNA at each time-point was performed as described above for the steady-state eNOS mRNA quantification. The fold-change in eNOS mRNA abundance at each time-point was determined by the equation:

$$\text{Fold change} = 2^{-\Delta C_T}, \text{ where } \Delta C_T = (C_{T,eNOS}) \text{ target} - (C_{T,eNOS}) \text{ 0 h}.$$

Pulse-chase experiments. Endothelial cells were cultured as described above followed by serum deprivation for an additional 2 h in cysteine-, methionine-, and serum-free

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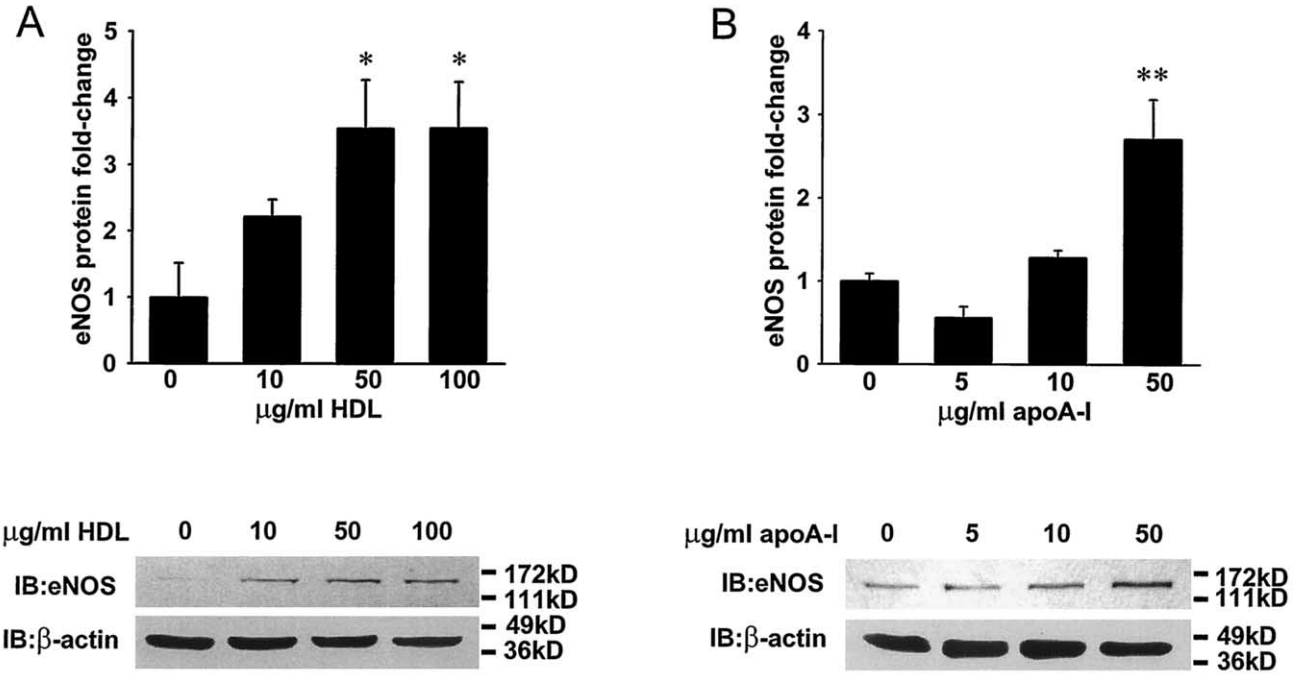

Figure 1. Both high-density lipoprotein (HDL) and apolipoprotein A-I (apoA-I) increase endothelial nitric oxide synthase (eNOS) protein abundance in cultured human vascular endothelial cells. **(A)** Human vascular endothelial cells were treated with HDL at concentrations from 0 to 100 μg/ml. After 24 h of incubation, cells were lysed and the expressions of eNOS and β-actin were analyzed by immunoblotting and spot densitometry. The HDL increased the abundance of eNOS protein up to 3.5 ± 0.7-fold at a concentration of 50 μg/ml ($p < 0.05$ vs. control). No further increase occurred at higher concentrations of HDL ($p = 0.993$ 50 μg/ml vs. 100 μg/ml of HDL). **(B)** Purified human apolipoprotein A-I (apoA-I) was added to the HAEC cultures at concentrations from 0 to 50 μg/ml, and the effect of apoA-I on eNOS protein abundance was analyzed as described for HDL. The apoA-I increased the amount of eNOS by 2.7 ± 0.5-fold ($p = 0.004$ vs. control, $n = 3$). At lower doses, no significant difference existed between apoA-I–treated cells and controls. Data presented in the **upper panels** are mean ± SEM of three independent experiments. **Lower panels** show a representative experiment. *$p < 0.05$ vs. control; **$p < 0.01$ vs. control.

DMEM (Invitrogen, Carlsbad, California) and labeled for 1 h with a mixture of [^{35}S]-methionine and [^{35}S]-cysteine at the specific radioactivity of 50 μCi/ml in the presence or absence of HDL (50 μg/ml). Cells were then rinsed five times and incubated for up to 10 h with HDL (50 μg/ml) or vehicle, in medium containing 300 mg/l of nonradioactive L-methionine and 630 mg/l of L-cysteine. Cells were lysed and eNOS protein was immunoprecipitated using monoclonal anti-eNOS antibody (Transduction Laboratories). Immunopellets were subjected to SDS–PAGE, and eNOS bands were detected using a phospho-imager. Parallel gels were blotted onto nitrocellulose membranes, and membranes were probed for eNOS as described above to ensure correct indentification of the eNOS protein band.

Immunocytochemistry. The EA.hy926 cells were incubated with HDL (50 μg/ml) and vehicle control for 0, 1, 3, and 24 h. Cells were fixed with 3.7% paraformaldehyde in PBS and then permeablized with 0.3% Triton X-100. Cells were stained with eNOS monoclonal IgG1 (1:50 dilution), and Caveolin-1 polyclonal (1:100) primary antibodies, purchased from BD Transduction Laboratories, and secondary antibodies FITC-conjugated donkey anti-mouse IgG (1:50), and Cy3-conjugated donkey anti-rabbit IgG (1:500), both obtained from Jackson ImmunoResearch, West Grove, Pennsylvania. Single and merged images were prepared using a Nikon OptiPhot-2 light microscope with fluorescent attachment and digital camera.

Signal transduction pathway experiments. The HAEC, cultured as described above, were harvested after exposure to HDL (50 μg/ml), apoA-I (50 μg/ml) or PBS-control for 0 to 24 h. The abundance of pERK1/2 or pAkt at each time-point was quantified by immunoblotting and spot densitometry. The density of the pERK1/2 or pAkt was normalized against total ERK1/2 or total Akt values, respectively.

In a subset of experiments, an inhibitor of MEK1-mediated phosphorylation of ERK1/2, PD98059 (Calbiochem) or LY294002, an inhibitor of PI3K-mediated phosphorylation of Akt (Sigma-Aldrich), was added to the cultures at concentrations ranging from 0 to 50 μmol/l. Cells were then pre-incubated for 30 min with the inhibitors before stimulation with HDL or vehicle. Incubation was continued for 0 to 24 h and the expressions of eNOS, beta-actin, pERK1/2, total ERK1/2, pAkt, and total Akt were analyzed by immunoblotting.

Statistical analysis. Pair-wise comparisons were performed with a Student *t* test. Multiple comparisons were performed using one-way analysis of variance (ANOVA) followed post hoc by the Student-Newman-Keuls test. Data are expressed as mean ± SEM unless otherwise indicated. A $p \leq 0.05$ was

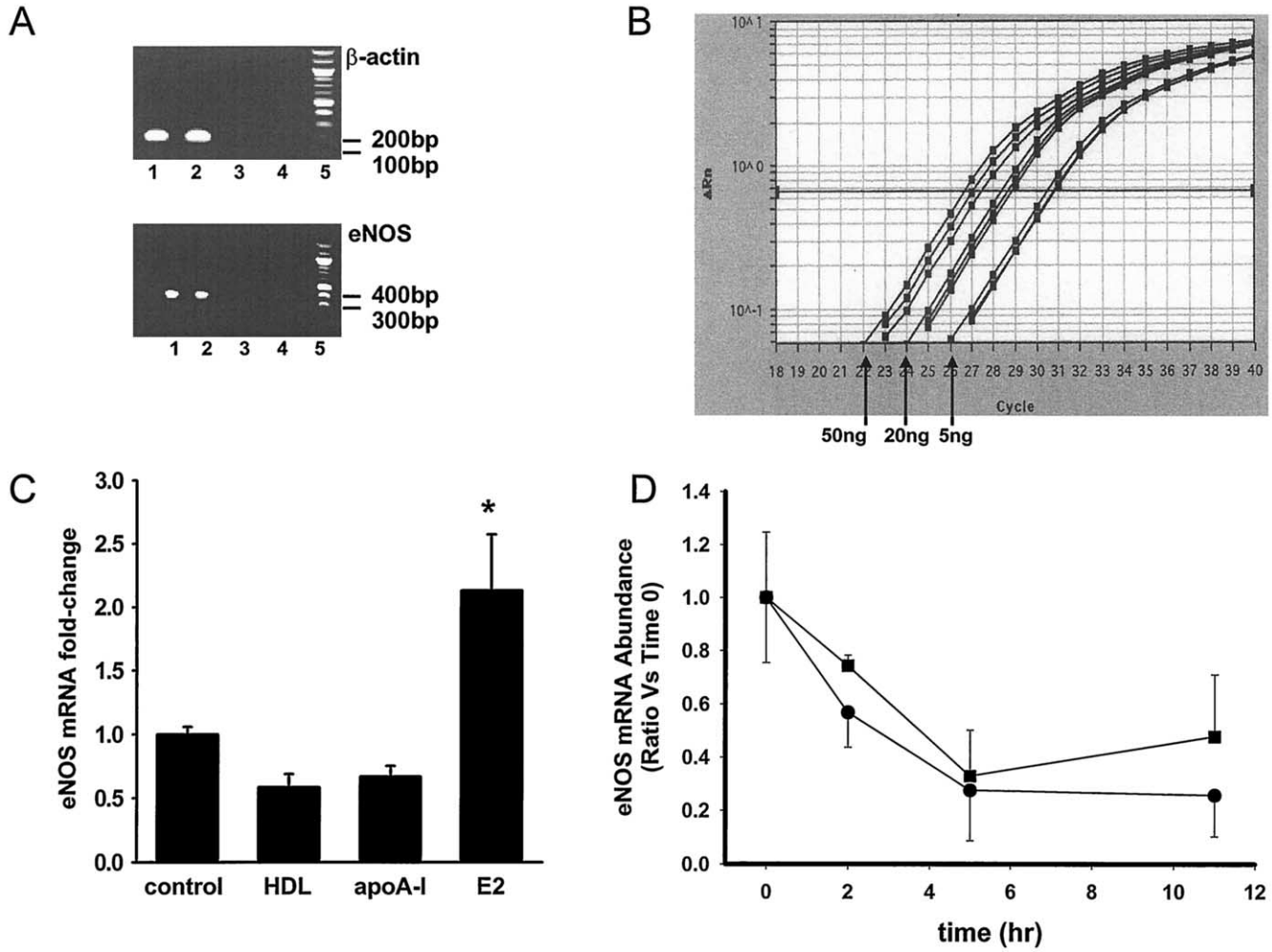

Figure 2. Neither HDL nor apoA-I increases eNOS messenger ribonucleic acid (mRNA) abundance in human vascular endothelial cells. **(A)** Ethidium bromide gel analysis of real-time quantitative polymerase chain reaction (RT–PCR) products demonstrating that only one band of the appropriate size was visible after amplification of eNOS and β-actin mRNA. **Lanes 1 and 2** = positive control; **lane 3** = no reverse transcriptase; **lane 4** = no template; **lane 5** = 100 bp deoxyribonucleic acid-ladder. **(B)** A logarithmic amplification plot of standards for eNOS mRNA. The sets of plots represent data from amplification of 5, 20, and 50 ng of standard cDNA plotted as fluorescence signal (ΔRn) versus cycle number. N = 3 repetitive measurements of each concentration are shown. **(C)** The effect of HDL, apoA-I, and 17-β-estradiol on eNOS mRNA abundance in human vascular endothelial cells. Cells were treated with either HDL (50 μg/ml), apoA-I (50 μg/ml), 17-β-estradiol (10 nmol/l), or vehicle control for 24 h. Total cellular ribonucleic acid was reverse-transcribed, and 10 ng of resulting complementary deoxyribonucleic acid was subjected to the real-time PCR analysis. Relative changes in eNOS mRNA level were calculated as described in Materials and Methods. Neither HDL nor apoA-I increased eNOS mRNA abundance ($p = 0.093$ and $p = 0.145$, respectively vs. control, n = 4). Treatment with estradiol resulted in a 2.1 ± 0.4-fold induction in eNOS mRNA level ($p < 0.001$ vs. control, n = 4). Data are presented as mean ± SEM. *$p < 0.001$ vs. control. **(D)** The effect of HDL on eNOS mRNA stability. The eNOS mRNA abundance was determined in actinomycin D–treated cells at various time-points after HDL **(circles)** or phosphate-buffered saline (PBS) **(squares)** treatment. The HDL had no significant effect on eNOS mRNA half-life (n = 3; $p = 0.5$). Other abbreviations as in Figure 1.

regarded as statistically significant. All statistical calculations were performed using Sigmastat version 2.03.

RESULTS

Both HDL and apoA-I increase eNOS protein abundance in human vascular endothelial cells. Either EA.hy926 cells or HAEC were cultured as described above and incubated with HDL (0 to 100 μg/ml). A small amount of eNOS protein was detectable at baseline. The HDL increased eNOS protein abundance up to 3.5 ± 0.7-fold at a concentration of 50 μg/ml after 24 h of incubation ($p < 0.05$ vs. control, n = 3; Fig. 1A). At higher concentrations of HDL, no further increase occurred in eNOS abundance ($p = 0.993$ 50 μg/ml vs. 100 μg/ml of HDL, n = 3), and no significant increase in eNOS was observed at earlier time-points (data not shown). Similarly, at a dose of 50 μg/ml, apoA-I increased eNOS protein abundance in HAEC by 2.7 ± 0.5-fold ($p = 0.004$ vs. PBS control, n = 3; Fig. 1B). At doses of 5 and 10 μg/ml of apoA-I, no significant difference existed in eNOS abundance compared to the control cells ($p = 0.26$ and 0.45, respectively).

Neither HDL nor apoA-I increases eNOS mRNA levels in human vascular endothelial cells. We next used RT–PCR to determine whether HDL increases eNOS protein abundance by increasing eNOS mRNA level. In preliminary experiments, PCR reactions resulted in a single product of

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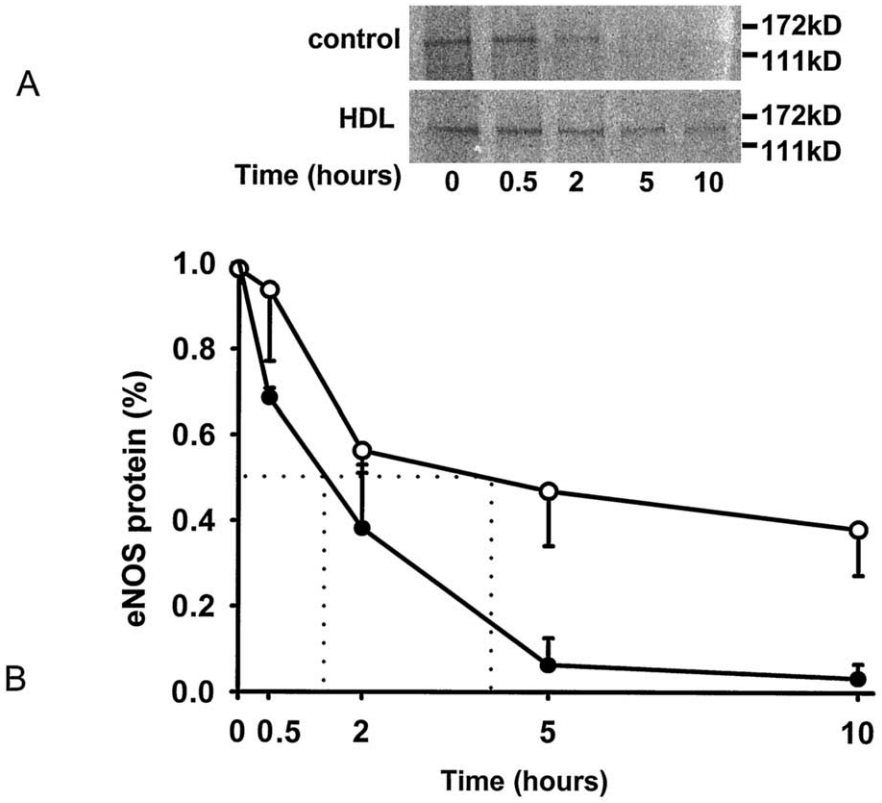

Figure 3. HDL increases eNOS protein half-life in human vascular endothelial cells. **(A)** Autoradiograph showing immunoprecipitated eNOS protein from a representative pulse-chase experiment after the indicated "chase periods." **(B)** The averaged results of three pulse-chase experiments are shown. **Open circles** represent cells treated with HDL (50 μg/ml) and **solid circles** represent control cells. Incubation with HDL increased eNOS protein half-life from 81 ± 0.1 to 270 ± 24 min ($p = 0.001$ vs. control, $n = 3$). Data presented in B are mean ± SEM; $n = 3$ independent experiments. Abbreviations as in Figure 1.

the expected size for both beta-actin and eNOS (Fig. 2A). Serial dilutions of control cDNA were used to demonstrate that the fold-induction in mRNA levels correlated linearly with the amount of input cDNA (Fig. 2B). After validating the assay, we then analyzed the effect of HDL (50 μg/ml) and apoA-I (50 μg/ml) on eNOS mRNA abundance. Surprisingly, neither HDL nor apoA-I significantly affected eNOS mRNA levels (Fig. 2C). As expected, 17-beta-estradiol significantly increased eNOS mRNA 2.1 ± 0.06-fold ($p < 0.001$ vs. control, $n = 4$; Fig. 2C). To determine the effects of HDL on eNOS mRNA degradation, quantitative RT–PCR analyses of eNOS mRNA abundance were carried out in EA.hy926 cells pretreated with actinomycin D. The half-life of eNOS mRNA was not significantly changed in the presence of 50 μg/ml HDL (2.9 ± 0.6 h) compared to PBS (3.6 ± 0.5 h; Fig. 2D; $n = 3$; $p = 0.5$).

HDL increases eNOS protein stability. Pulse-chase experiments were performed next to determine whether HDL increases eNOS protein abundance by increasing the half-life of eNOS protein. The HDL slowed the degradation of eNOS protein by 3.3 ± 0.2-fold, increasing its half-life from 81 ± 0.1 min to 270 ± 24 min ($p = 0.001$ vs. control, $n = 3$; Fig. 3). Immunocytochemical studies were next performed to determine whether the HDL-induced increase in eNOS protein stability was accompanied by alterations in the intracellular localization of the protein. In unstimulated cells, HDL co-localized with caveolin-1. After 3 h of HDL treatment, eNOS translocated to a predominantly peri-nuclear location, but then returned to baseline by 24 h (Fig. 4). In contrast, PBS had no effect on eNOS protein localization (Fig. 4).

Both HDL and apoA-I activate ERK1/2 and Akt in human aortic endothelial cells. We hypothesized that pathways regulating the enzymatic activity of eNOS might also participate in regulating its abundance over longer time periods. Therefore, as a first step in identifying the signaling pathways that mediate the HDL-induced increase in eNOS protein abundance, the effects of HDL on phosphorylation of ERK1/2 and Akt were examined. Previous reports have demonstrated that both MAP-kinase and Akt can regulate eNOS enzymatic activity (5–7). The HDL increased the amount of pERK1/2 in human arterial endothelial cells by 5.5 + 1.2-fold after 5 min of incubation ($p = 0.005$ vs. control, $n = 4$; Fig. 5), and by 8.3 ± 0.8-fold after 10 min of incubation ($p < 0.001$ vs. control, $n = 4$; Fig. 5). The activation was transient, because after 30 min of incubation the HDL-induced increase in pERK1/2 abundance had decreased to only 1.5 ± 0.13-fold ($n = 4$, $p = 0.02$), and after 3 h of incubation no significant difference existed between HDL and control samples ($p = 0.6$, $n = 4$; Fig. 5).

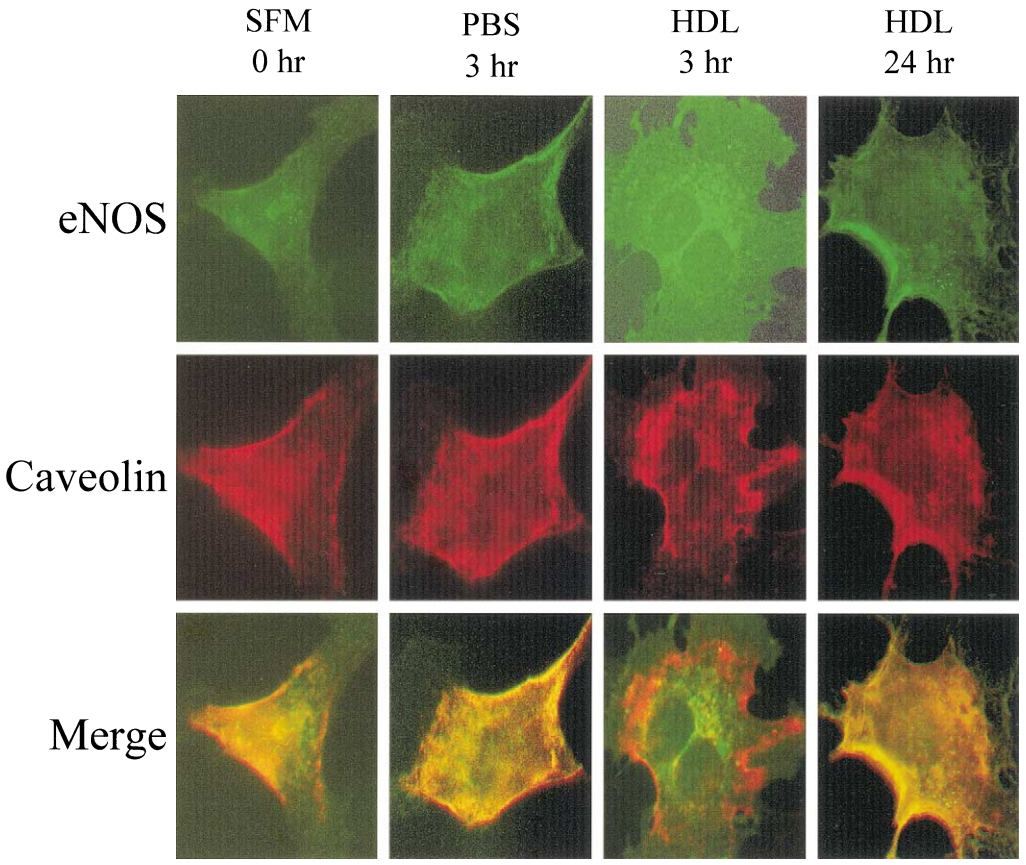

Figure 4. HDL causes transient migration of eNOS from the caveolae to a peri-nuclear distribution. The EA.hy926 cells were stained for both eNOS **(green)** or caveolin **(red)** in serum-free medium (SFM) or after stimulation with HDL (50 μg/ml) or PBS for 3 or 24 h. (Shown are representative images from one of three independent experiments.) Abbreviations as in Figure 1.

Similarly, apoA-I increased the abundance of pERK1/2 in human arterial endothelial cells by 2.3 ± 0.3-fold after 5 min of incubation ($p < 0.001$ vs. PBS, $n = 4$) and 1.8 ± 0.2-fold following 10 min of incubation ($p = 0.002$ vs. PBS, $n = 4$; Fig. 5). At later time-points, no significant difference was seen in pERK1/2 abundance between apoA-I and

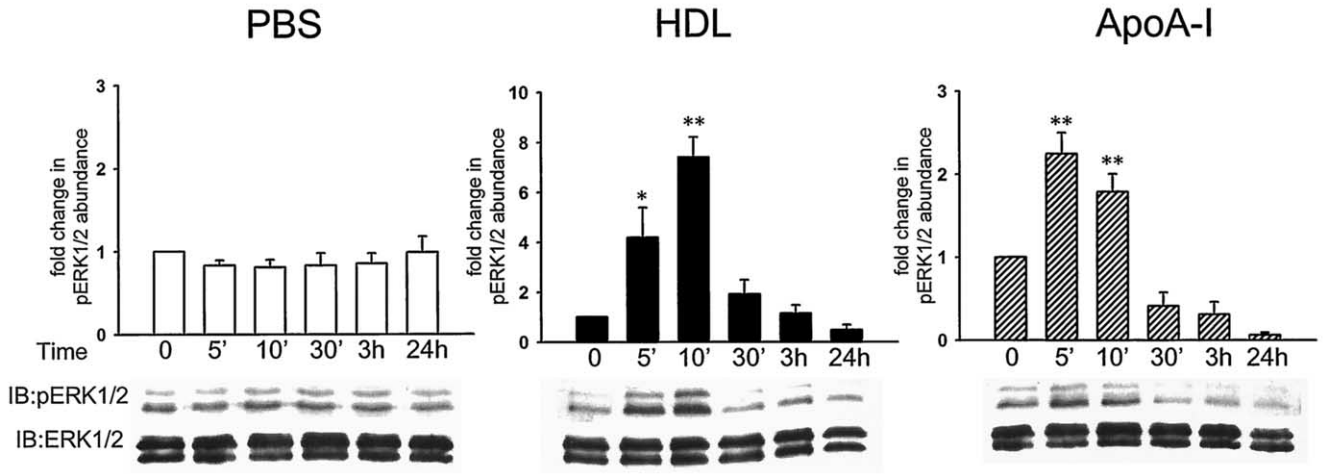

Figure 5. HDL and apoA-I increase ERK1/2 phosphorylation in human arterial endothelial cells. The HAEC treated with either HDL (50 μg/ml), apoA-I (50 μg/ml), or PBS were lysed at the indicated time-points and the amount of pERK1/2 and total ERK1/2 were measured by immunoblotting and spot densitometry. The density of pERK1/2 bands was normalized against the corresponding total ERK1/2 band. The HDL increased the abundance of pERK1/2 5.5 ± 1.2-fold ($p = 0.005$ vs. control) after 5 min of incubation and 8.3 ± 0.8-fold ($p < 0.001$ vs. control) after 10 min of incubation. The apoA-I increased the abundance of pERK1/2 2.3 ± 0.3-fold after 5 min of incubation ($p < 0.001$ vs. control). Data shown are mean ± SEM, $n = 4$ independent experiments. The **lower panels** depict a representative blot of each treatment. *$p < 0.005$ vs. control; **$p < 0.001$ vs. control. Abbreviations as in Figure 1.

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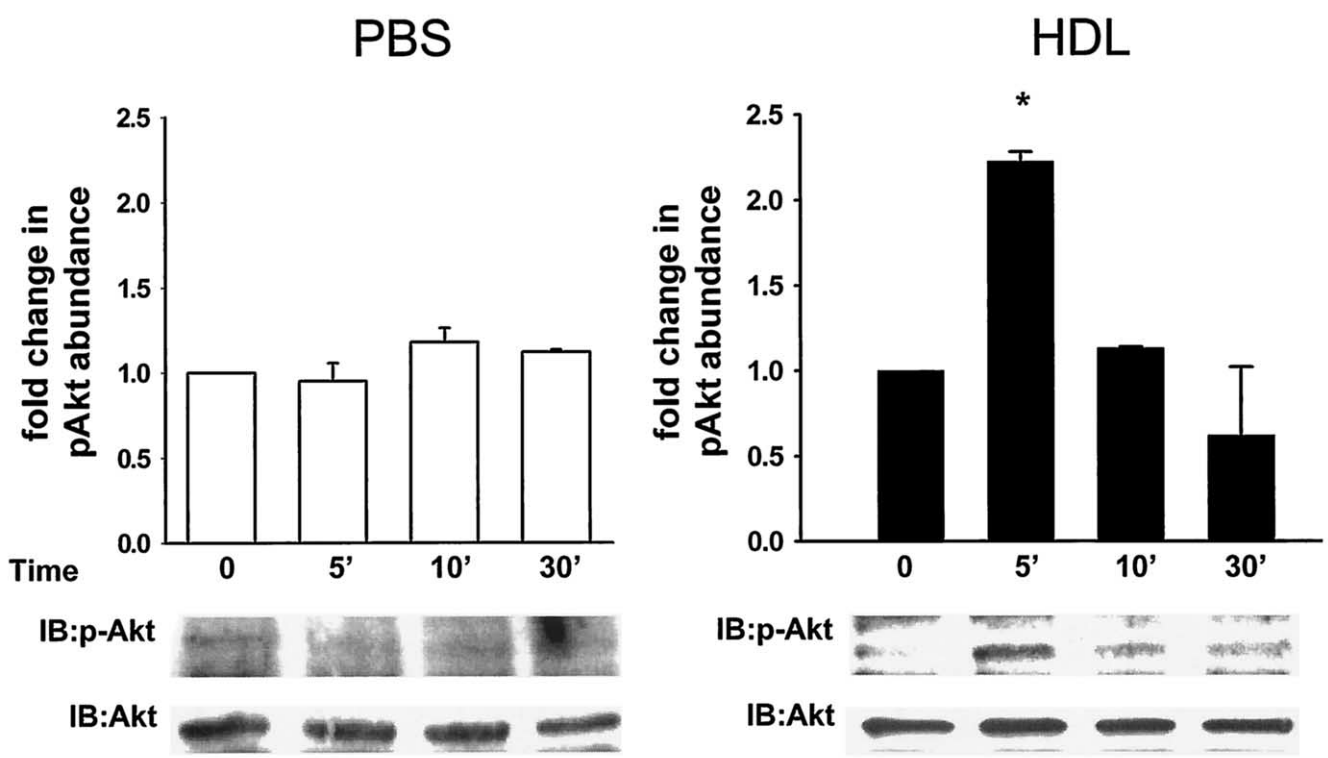

Figure 6. HDL increases Akt phosphorylation in human vascular endothelial cells. Cells were treated with HDL or PBS, lysed at the indicated time-points, and the amount of pAkt and total Akt was measured by immunoblotting and spot densitometry. The density of the pAkt band was normalized against total Akt. After 5 min of incubation, HDL increased the amount of pAkt in human vascular endothelial cells by 2.2 ± 0.05-fold ($p < 0.001$ vs. control). Data are shown as mean ± SEM, n = 3 independent experiments. **Lower panels** depict a representative blot of each treatment. *$p < 0.001$ vs. control. Abbreviations as in Figure 1.

control samples. Also, HDL transiently increased the amount of pAkt in human vascular endothelial cells by 2.2 ± 0.05-fold at 5 min ($p < 0.001$ vs. control, n = 3; Fig. 6). The amounts of total ERK1/2 and Akt were not altered by any of these stimuli over the time period examined.

Inhibition of either ERK1/2 or Akt activation inhibits the HDL-induced increase in eNOS protein abundance. The effect of pharmacologic inhibition of activation of ERK1/2 and Akt on HDL-induced effects on eNOS protein abundance was examined next. Incubation of cultures with PD98059, a specific inhibitor of MEK1-mediated ERK1/2 activation, dose-dependently inhibited the transient HDL-induced phosphorylation of ERK1/2, with complete inhibition of ERK1/2 phosphorylation achieved at 50 μmol/l (Fig. 7A). Pretreatement with 50 μmol/l PD98059 also significantly blocked the effect of HDL on eNOS protein abundance ($p = 0.029$ between HDL and HDL + 50 μmol/l PD98059, n = 3).

Similarly, LY294002, an inhibitor of PI3K–mediated activation of Akt, dose-dependently inhibited the transient HDL-induced Akt phosphorylation (Fig. 7B), and this too blocked the HDL-induced increase in eNOS protein abundance ($p < 0.001$ vs. control, n = 3; Fig. 7B). Because inhibition of either the PI3K/Akt or MAP-kinase pathways alone was sufficient for inhibiting the effect of HDL on eNOS protein abundance, the specificity of each of the inhibitors for their respective pathways was examined. As shown in Figure 7C, LY294002 did not inhibit HDL-induced ERK1/2 phosphorylation, and PD98059 did not inhibit HDL-induced Akt phosphorylation under these experimental conditions.

DISCUSSION

The eNOS-dependent production of nitric oxide (NO) is known to be regulated by two basic mechanisms. The better studied of these involves alterations in the enzymatic activity of the protein that occur rapidly in response to a variety of stimuli, including HDL, which has been described to rapidly activate eNOS in Chinese hamster ovary cells (14,15). More recently it has also been clearly demonstrated that eNOS protein expression can be both up- or down-regulated by various stimuli, including estradiol (16), cyclic strain (17), and HMG CoA reductase inhibitors (12). According to current knowledge, each of these stimuli increases eNOS protein abundance by increasing eNOS mRNA levels, either by upregulating gene transcription or by stabilizing mRNA (reviewed in Govers and Rabelink [18]). In the current study, we demonstrate that HDL and apoA-I also increase the amount of eNOS protein in cultured human vascular endothelial cells. The different dose-responses observed between HDL and apoA-I suggest

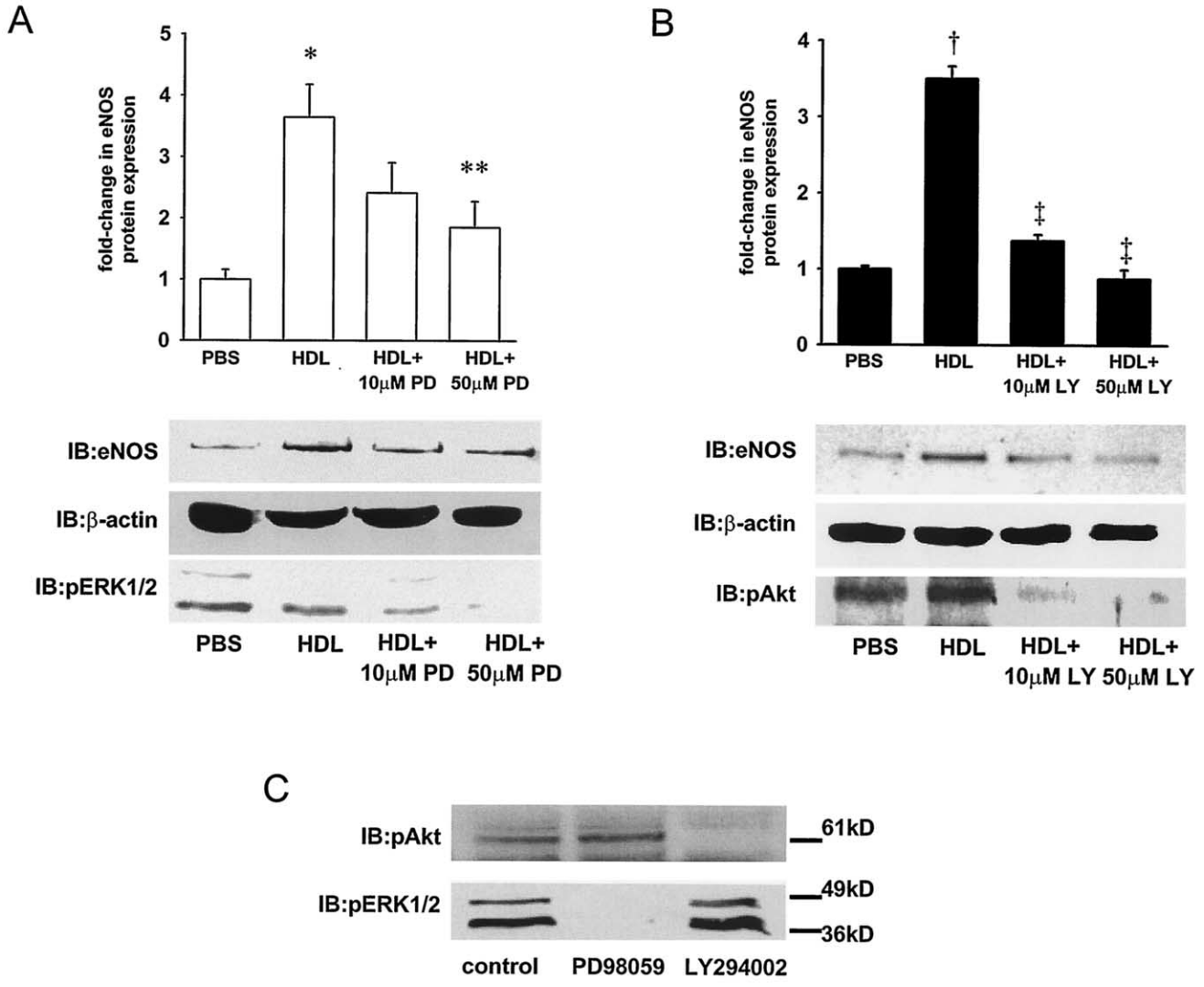

Figure 7. Both PD98059 and LY294002 abolish the HDL-induced increase in eNOS protein abundance. **(A)** HDL increased the abundance of eNOS by 3.6 ± 0.53-fold ($p = 0.006$ vs. PBS), and 50 μmol/l PD98059 significantly inhibited this increase ($p = 0.029$ vs. HDL). **(B)** HDL increased eNOS protein abundance by 3.5 ± 0.17-fold ($p = 0.001$ vs. PBS), and LY294002 inhibited this increase dose-dependently and significantly at concentrations of 10 and 50 μmol/l ($p < 0.001$ vs. HDL, $n = 3$). Data presented are mean ± SEM, $n = 3$ independent experiments. Lower panels depict a representative blot. **(C)** Cells preincubated for 30 min in either PD98059 or LY294002 were stimulated with HDL for 5 min. Cells were lysed and the amount of pERK1/2 and pAkt was then analyzed by Western blotting. The LY294002 did not inhibit HDL-induced ERK1/2 activation. Similarly, PD98059 did not inhibit HDL-induced Akt activation. A representative blot of $n = 3$ is shown. *$p < 0.01$ vs. control; **$p < 0.001$ vs. HDL; †$p < 0.001$ vs. PBS; ‡$p < 0.001$ vs. HDL. Abbreviations as in Figure 1.

that other components of the HDL particle may also contribute to the observed response. Surprisingly, however, HDL had no significant effect on eNOS mRNA levels or half-life. Rather, HDL increased the half-life of eNOS protein, apparently by decreasing degradation of the protein. Moreover, HDL treatment caused eNOS to transiently adopt a peri-nuclear distribution, though the significance of this in terms of its effect on eNOS half-life remains unclear. Taken together, these data support that, unlike other stimuli known to upregulate eNOS protein abundance, HDL increases eNOS protein abundance via a posttranslational mechanism.

We next sought to identify signaling pathways that contribute to HDL's effect on eNOS protein abundance. We hypothesized that the same pathways that rapidly increase the enzymatic activity of eNOS might also initiate the events necessary to lead to longer-term accumulation of eNOS protein. Therefore, we examined whether HDL alters the activity of two kinase-dependent signaling pathways that have previously been shown to regulate eNOS activity, and we also sought to determine whether inhibition of these pathways blocks HDL-mediated effects on eNOS protein abundance. The current findings are consistent with previous reports demonstrating that HDL, at physiologically relevant concentrations, transiently enhances phosphorylation of both MAP-kinase and Akt on sites previ-

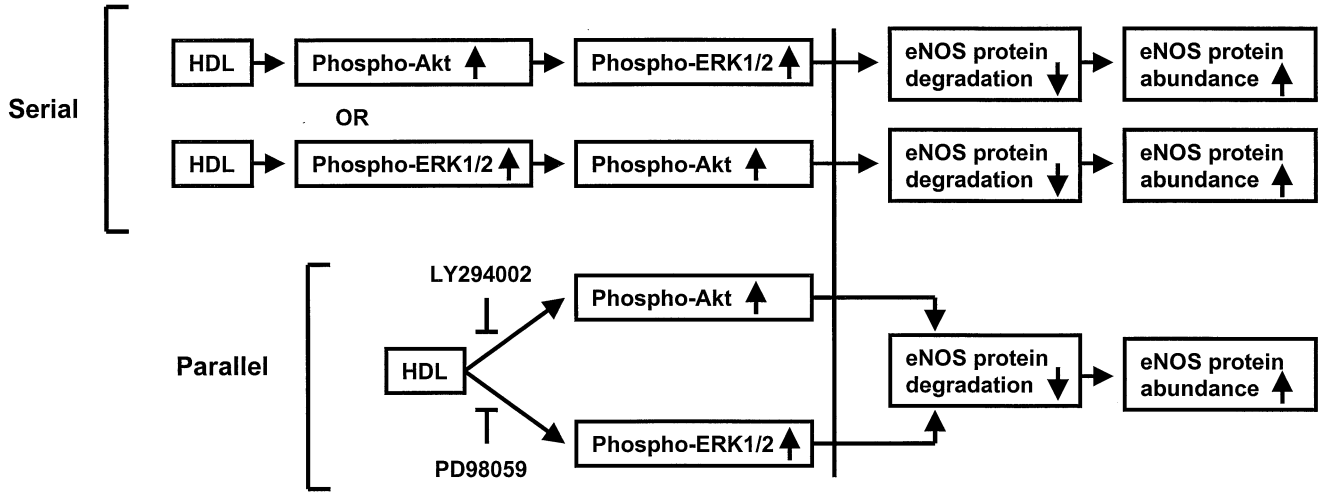

Figure 8. Potential pathways by which HDL increases eNOS protein abundance in a kinase-dependent manner. In the **upper portion** of the figure, two potential serial kinase pathways are shown. In the **lower panel,** parallel arrangements of kinases are shown in which activation of both pathways is required to alter eNOS abundance. The observation that pharmacologic inhibition of either kinase cascade alone blocks HDL-induced increases in eNOS abundance favors the parallel model. Abbreviations as in Figure 1.

ously shown to correlate with enhanced kinase activity of each protein. Furthermore, pharmacologic inhibition of HDL-mediated activation of either of these pathways alone completely blocked HDL-induced increases in eNOS protein abundance. As shown in Figure 8, this suggests two hypotheses: 1) that MAP-kinase and Akt are arranged serially in the pathway that regulates eNOS protein stability, such that inhibition of either kinase blocks this effect, or 2) that MAP-kinase and Akt act via parallel pathways, but activation of both pathways is required to enhance the stability of eNOS. The observation that each of the kinase inhibitors used in these studies results in inhibition of only the anticipated pathway (Fig. 7C) indicates that the parallel pathway model is more likely to be true. Though these findings clearly implicate HDL-mediated activation of MAP-kinase and Akt in the effect of HDL on eNOS protein abundance, further investigation will be required to determine the downstream effects of kinase cascade activation, which subsequently alters eNOS protein stability.

Study limitations. Several limitations of the current findings deserve mention. First, we did not directly measure enzymatic activity of the kinases of interest. However, the phospho-forms identified by this technique have previously been shown to correlate with enzyme activation (19,20). Second, the data presented demonstrate that inhibition of the HDL-induced rapid, transient increase in kinase phosphorylation also blocked HDL's effect on eNOS protein abundance, but they do not elucidate the downstream effects that mediate this. A number of mechanisms can be envisioned by which these early transient effects could lead to delayed, longer-term effects. The kinases could directly alter eNOS protein phosphorylation, which in turn alters interactions with other proteins that regulate its degradation. Both MAP-kinase and Akt have previously been shown to alter the activity of transcription factors, and this could lead to altered levels of a protein that then regulates eNOS protein degradation. Clearly distinguishing between these possibilities will require extensive additional experimentation. Finally, we did not directly investigate effects of HDL on eNOS gene transcription.

Conclusions. In conclusion, we have demonstrated that HDL activates both ERK1/2 and Akt, resulting, by an unknown mechanism, in enhanced eNOS protein stability and subsequent accumulation of eNOS protein. This post-translational regulation represents a previously unrecognized mechanism for regulating eNOS expression in the endothelium. Taken together with our previous observation that raising HDL in patients with CAD enhances endothelium-dependent vasorelaxation (2), the current findings further support an important role of HDL in regulating vascular function.

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